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FINAL REPORT

USP ANTIMICROBIAL PRESERVATIVE EFFECTIVENESS TEST

PROCEDURE NO. STP0131 REV 02
PROTOCOL DETAIL SHEET NO. 200801838 REV 01

LABORATORY NO. 429606

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QAU AUDIT STATEMENT

[X] USFDA (21 CFR PART 58) [ ] USEPA (40 CFR PART 160)

USP ANTIMICROBIAL PRESERVATIVE EFFECTIVENESS TEST

LABORATORY NO. 429606

1. The test was conducted in accordance with the USFDA or USEPA Regulations as noted above.

2. In accordance with the Good Laboratory Practice Regulations, the Assaying and Plating phase(s) of this study was inspected by the Quality Assurance Unit on: 28 Jul 2008. The findings of the inspection(s) were reported to the Study Director on: 31 Jul 2008 and to Management on: 01 Aug 2008.

3. The Quality Assurance Unit has reviewed this report and has determined that the methods and standard testing procedures are accurately described, and that the reported results accurately reflect the raw data.

4. The name of the study director, the names of other scientists or professionals, and the names of all supervisory personnel, involved in the study:

Michael Neilson
Peter Croci
Dr. Jerry Nelson
Jeff Hills

QUALITY ASSURANCE: David Montag DATE: 01 Aug 2008

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USP ANTIMICROBIAL PRESERVATIVE EFFECTIVENESS

LABORATORY NUMBER:	429606
PROCEDURE NUMBER:	STP0131 REV 02
PROTOCOL DETAIL SHEET NUMBER:	200801838 REV 01
SAMPLE SOURCE:	American Biotech Labs
SAMPLE IDENTIFICATION:	Refer to Tables 1-2
DEVIATIONS:	None
PROTOCOL APPROVAL DATE:	24 Jun 2008
SAMPLE RECEIVED DATE:	10 Jun 2008
LAB PHASE START DATE:	24 Jun 2008
LAB PHASE COMPLETION DATE:	01 Aug 2008
REPORT ISSUE DATE:	01 Aug 2008

INTRODUCTION:

This study was performed to determine the survival rate of various organisms in the test product. The test employed methods designed to determine antimicrobial effectiveness described in the United States Pharmacopeia (USP).

The samples of the product were inoculated, in duplicate, with five standard test organisms. The inoculated samples were then incubated for a total of 28 days at 20-25°C. Aliquots from the samples were immediately removed and assayed for surviving organisms at 0 hour, 7, 14 and 28 day time intervals. The log reduction in the level of the test organisms was calculated for each time interval.

PROCEDURE:

The following organisms were tested:

1) *Staphylococcus aureus* ATCC #6538
   [Bacteria, Gram (+) cocci]
2) *Pseudomonas aeruginosa* ATCC #9027
   [Bacteria, Gram (-) bacillus]
3) *Escherichia coli* ATCC #8739
   [Bacteria, Gram (-) bacillus]
4) *Candida albicans* ATCC #10231
   [Yeast]
5) *Aspergillus niger* ATCC #16404
   [Mold]

The bacteria were transferred to soybean casein digest agar (SCDA) and incubated at 30-35°C for 18-24 hours. The *C. albicans* and *A. niger* were transferred to Sabouraud dextrose agar (SDEX). The *C. albicans* was incubated at 20-25°C for 44-52 hours and the *A. niger* was incubated at 20-25°C for 6-10 days.

The bacteria and *C. albicans* were harvested using 0.9% saline and *A. niger* was harvested with 0.9% saline containing 0.05% Tween® 80 (SALT). The *A. niger* was filtered through gauze and all cultures were vortexed to break up clumps. The titer of each suspension was adjusted to approximately 10^8 colony forming units (CFU)/mL using visual turbidity. The actual titer of each culture was determined using the positive control values.

Two 7.0 mL samples of the test product were prepared for each challenge organism. The duplicate tubes containing 7.0 mL were then inoculated with 70 µL of the test organism using a calibrated micropipettor. The volume of the inoculum was between 0.5% and 1.0% of the volume of the product. The final concentration of the test preparation was approximately 10^5 - 10^6 CFU/mL of product. The samples were well mixed.

Two positive control tubes were prepared for each organism using sterile water. Negative controls were also prepared. A 70 µL aliquot of the test organism was added to 7.0 mL of sterile water for the positive control. All test samples were stored at 20-26°C for a total of 28 days.

Control tubes and test vials were assayed immediately to determine the initial concentration of organisms in each tube. The test suspensions were assayed at the following intervals: 0, 7, 14 and 28 days.

Sample aliquots at each interval were diluted in letheen broth (LETH) and plated on SCDA for bacteria and SDEX for *C. albicans* and *A. niger*. The bacteria plates were incubated at 30-35°C for 3-5 days. The *C. albicans* plates were incubated at 20-25°C for 3-5 days and the *A. niger* plates were incubated at 20-25°C for 3-7 days.

NEUTRALIZATION JUSTIFICATION:

Additional controls were performed to ensure neutralization. This was performed by adding 1.0 mL of un-inoculated product to 9.0 mL of LETH. This simulates the highest concentrations of the product plated, and represents the worst case for neutralization. The tubes were then inoculated with 0.1 mL of a 1,000-10,000 CFU/mL organism suspension, and plated in 0.5 mL aliquots on the appropriate recovery agar. Control tubes of LETH, without any product, were inoculated concurrently with the same known cultures and plated. Neutralization is demonstrated when the number of colonies on the neutralized sample plates demonstrates at least 70% recovery when compared to the control plates and all plate counts show a recovery of ≤ 100 CFU/plate.

Letheen broth (the neutralizer used) is a very common neutralizing broth and has not been shown to inhibit growth of the 5 standard organisms used for this test method. In addition, the 5 samples (1 per organism) and the 5 positive controls are diluted in the same neutralizing broth, and then plated. Any inhibitory effect that could be present would affect both the positive control recoveries as well as the sample recoveries, thus final log reduction results would not be affected by any possible toxicity of the neutralizer broth used.

ACCEPTANCE CRITERIA:

According to USP, products are placed into categories according to product type. The product category will determine the acceptance criteria for the test. The following table provides a description of the current USP categories.

CATEGORY	PRODUCT DESCRIPTION
1	Injections, other parenterals including emulsions, otic products, sterile nasal products, and ophthalmic products made with aqueous bases or vehicles.
2	Topically used products made with aqueous bases or vehicles, non-sterile nasal products, and emulsions, including those applied to mucous membranes
3	Oral products other than antacids, made with aqueous bases or vehicles.
4	Antacids made with an aqueous base.

On the following page is the acceptance criteria for the USP Antimicrobial Effectiveness Test. No increase is defined as not more than a 0.5 $\log_{10}$ unit higher than the previous value measured.

Category 1 Products	
Bacteria:	Not less than 1.0 log reduction from the initial calculated count at 7 days, not less than 3.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast & Molds:	No increase from the initial calculated count at 7, 14, and 28 days.

Category 2 Products	
Bacteria:	Not less than 2.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast & Molds:	No increase from the initial calculated count at 14 and 28 days.

Category 3 Products	
Bacteria:	Not less than 1.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast & Molds:	No increase from the initial calculated count at 14 and 28 days.

Category 4 Products	
Bacteria, Yeast,& Molds:	No increase from the initial calculated count at 14 and 28 days.

RESULTS:

The results reported in Table 1 are representative of two replicates plated in duplicate. The greater than (>) values represent the detectable limits of the test where zero CFU were observed on the plates. The approximate (~) symbol is applied to results where plate counts fell outside of the statistically accurate range of 25-250 CFU.

The neutralization data is found in Table 2. The neutralization testing showed adequate neutralization of all products tested at the 1:10 dilution when challenged against the standard test organisms.

All negative controls showed no growth.

CONCLUSION:

Interpretation of the data is the responsibility of the sponsor and no conclusion can be made by Nelson Laboratories, Inc. (NLI).

DATA DISPOSITION:

The raw data and final report from this study are archived at NLI or an approved off-site location.

STATEMENT OF UNCERTAINTY:

If applicable, the statement of uncertainty is available to sponsors upon request.

Peter Croel
Study Director

01 Aug 2007
Study Completion Date

MJN/es

TABLE 1. Summary of Log Reduction Results
Sample Identification: AGX Wound Care Lot #060908

ORGANISM	TIME INTERVAL			
	0 HOUR	7 DAY	14 DAY	28 DAY
S. aureus	0.91	>4.72	>4.72	>4.72
P. aeruginosa	>3.31	>4.31	>4.31	>4.31
E. coli	>3.12	>4.56	>4.56	>4.56
C. albicans	>3.73	>4.73	>4.73	>4.73
A. niger	-0.19	>3.59	>3.59	>3.59

TABLE 2. Neutralization Data

SAMPLE IDENTIFICATION	ORGANISM	CONTROL	SAMPLE	PERCENT OF CONTROL (%)
AGX Wound Care Lot #060908	*S. aureus*	27	31	115
	P. aeruginosa	16	12	75
	E. coli	29	25	86
	C. albicans	50	40	80
	A. niger	7	6	86

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1.0 INTRODUCTION

1.1 Purpose: This test is designed to determine the effectiveness of an antimicrobial preservative. Antimicrobial preservatives are substances which are commonly added to multiple dose containers to protect them from microbial contamination and inhibit the growth of the test microorganisms that may be inadvertently introduced during use. The procedure involves the contamination of the test system with known numbers of standard laboratory strains selected to provide a diverse challenge to the system. At periodic intervals samples are removed and assayed to determine survival. Interpretation of the results is based on log reduction and survivor data.

1.2 Scope: This procedure is appropriate for testing products that contain an antimicrobial agent or that are otherwise inhibitory to the test organisms. It is a quantitative test that allows the determination of the amount of organism reduction at pre-determined intervals.

1.3 Justification: Multiple dose containers and products, which include chemical sterilization systems, must be evaluated for their ability to stabilize or reduce the manufacturing or adventitious in-use contaminants. This test is a severe challenge. The procedure was written to comply with the United States Pharmacopeia (USP) in content and intent.

2.0 REFERENCES

2.1 United States Pharmacopeia 31 & National Formulary 26. 2008. <51> Antimicrobial *Effectiveness. United States Pharmacopeial Convention, Inc., Rockville, MD. (CRD266)*

3.0 RECORDS

3.1 FRM0268 – USP Antimicrobial Preservative Effectiveness Test Forms

3.2 FRM0435 – Neutralization Plate Count Results -Optional

4.0 RESPONSIBILITY

4.1 The Study Director maintains overall responsibility for conducting this study.

5.0 DEFINITIONS

5.1 APE – Antimicrobial Preservative Effectiveness.

5.2 NEUTRALIZER – Substance which renders inert the activity of antimicrobials.

5.3 USP – United States Pharmacopeia.

6.0 SAFETY

6.1 Lab coat and protective glasses are required at all times in the laboratory while testing is being performed.

6.2 Food or drink is not allowed in the laboratory.

7.0 FREQUENCY

7.1 This document is reviewed approximately biennially.

7.2 Changes to this procedure may be proposed at any time, as needed (i.e., process or test method changes, reference updates, etc.).

8.0 ACCEPTANCE CRITERIA

8.1 Positive and negative controls should be respectively positive and negative for growth.

8.2 Neutralization control must demonstrate a ≥ 70% organism recovery at the reported product dilution.

9.0 MATERIALS

9.1 Equipment

9.1.1 Incubator capable of 30-35°C, and 20-25°C

9.1.2 Refrigerator capable of 2-8°C

9.1.3 Vortex mixer

9.1.4 Balance sensitive to 0.001 g

9.1.5 Micropipettors

9.1.6 Pipette aids

9.2 Supplies

9.2.1 Sterile 1, 5, & 10 mL pipettes

9.2.2 Sterile test tubes with closures

9.2.3 Sterile bent glass rods

9.2.4 Sterile pipette tips

9.3 Reagents

9.3.1 0.9% Physiological saline solution (PHSS)

9.3.2 Saline Tween® (SALT)

9.3.3 Sterile Purified Water (PURW)

9.4 Media

9.4.1 MMF0264 – Soybean casein digest agar (SCDA)

9.4.2 MMF0274Sabouraud dextrose agar (SDEX)

9.4.3 MMF0199 – Soybean casein digest neutralizer agar (NUAG)

9.4.4 MMF0128 – Letheen broth (LETH)

9.4.5 Other neutralizer broth as appropriate

9.5 Microorganisms

9.5.1 *Staphylococcus aureus* ATCC #6538

9.5.2 *Pseudomonas aeruginosa* ATCC #9027

9.5.3 *Escherichia coli* ATCC #8739

9.5.4 *Candida albicans* ATCC #10231

9.5.5 *Aspergillus niger* ATCC #16404

10.0 CULTURE PREPARATION

10.1 Maintain test organisms lyophilized and stored below -15°C. The microorganisms used in testing must not be more than five passages removed from the original ATCC culture.

10.2 Transfer the bacteria to soybean casein digest agar (SCDA).

10.3 Transfer the *Candida albicans* and *Aspergillus niger* to Sabouraud dextrose agar (SDEX).

10.4 Incubate cultures as outlined in the following table:

ORGANISM	MEDIA	TEMP	TIME
Staphylococcus aureus	SCDA	30-35°C	18-24 hours
Pseudomonas aeruginosa	SCDA	30-35°C	18-24 hours
Escherichia coli	SCDA	30-35°C	18-24 hours
Aspergillus niger	SDEX	20-25°C	6-10 days
Candida albicans	SDEX	20-25°C	44-52 hours

arvest the bacteria and *C. albicans* cultures with PHSS. For maximum harvest, flood the plates and use a glass rod to suspend growth in the saline. Use the bacterial and yeast suspensions within 24 hours of harvest.

10.6 Harvest the *A. niger* culture similarly, but with saline Tween® (SALT), a solution of 0.9% saline with 0.05% polysorbate 80 (Tween®).

10.6.1 Remove the mycelial mats with a sterile spatula.

10.6.2 Filter the organism through several layers of sterile cotton or gauze to remove clumps and debris as needed.

10.6.3 Vortex the suspensions vigorously to further break up clumps.

10.6.4 The fungal preparation may be stored under refrigeration for up to 7 days.

10.7 Adjust the concentration of each suspension to approximately 10^8 CFU/mL. Refrigerate the suspensions if not used within 2 hours of preparation.

10.8 Immediately before or concurrent with the test, perform plate counts on each suspension used. The positive controls may serve as this titration.

11.0 TEST PROCEDURE

11.1 Prepare separate sample tubes for each test organism, mixing well to assure uniformity.

11.2 Inoculate each sample tube with the appropriate organism using a calibrated micropipettor. The volume of the inoculum should be between 0.5% and 1.0% of the volume of the product. The concentration of microorganisms that is added to the product should be such that the final concentration of the test preparation after inoculation is between 1×10^5 and 1×10^6 CFU/mL of product. For Category 4 products (antacids) the final concentration of the test preparation after inoculation is between 1×10^3 and 1×10^4 CFU/mL of product. Test the sample as a single replicate or in duplicate as requested by the sponsor.

11.3 Prepare control tubes for each culture with the same volumes of sterile water (PURW) as were used for the sample. Inoculate the control tubes with the same amount of inoculum as determined with the sample. Test the control tubes in the same number of replicates as the sample.

11.4 Assay the controls immediately.

11.5 Incubate all test samples at 20-25°C.

11.6 Assay each test sample at the following intervals: 0, 7, 14, and 28 days.

11.7 Inspect the test samples at each of the test intervals listed above and describe any irregular appearance.

11.8 Assay the samples using serial dilution in a neutralizer broth. Use standard pour-plate or spread-plate technique. Plate sample aliquots on SCDA for bacteria and SDEX for molds and yeasts. Modify the media as necessary by adding neutralizers to inactivate the product. Incubate the bacteria plates at 30-35°C for 3-5 days. Incubate *C. albicans* plates at 20-25°C for 3-5 days and *A. niger* plates at 20-25°C for 3-7 days. Perform plate counts in duplicate or triplicate as requested by the sponsor.

11.9 After incubating for the appropriate amount of time, count the plates and calculate the log reduction and the concentration of each microorganism during the test, using the values obtained from the zero hour controls. Calculate the log reductions using the following formula:

{log (initial concentration) - log (final concentration)}

Example: $\log(1 \times 10^6) - \log(1 \times 10^3) = 6 - 3 = 3$

11.10 Monitor replicate variation, as necessary, by calculating the $\log_{10}$ difference between replicates.

11.10.1 If the variation is greater than a 0.5 $\log_{10}$ difference, evaluate, and report each replicate separately instead of a combined average of the two replicates if appropriate.

11.11 Perform a negative control with each plating to assure the sterility of the dilution liquids used (i.e. PURW for the control samples and Neutralizer broth for the test samples). Plate aliquots of the diluent onto both SCDA and SDEX. Incubate the SCDA plates at 30-35 °C for 3-5 days. Incubate the SDEX plates at 20-25°C for 3-7 days. Score plates for growth or no growth of the test organisms.

12.0 NEUTRALIZATION

12.1 Dilute organism suspensions used in testing to approximately 1,000-10,000 CFU/mL.

12.2 Prepare one tube per organism with 9 mL of LETH or other appropriate neutralizer broth and 1 mL/g product. This demonstrates the "worst-case" for neutralization. Alternately, further serial dilutions may be made if neutralization is not achieved at the first dilution.

12.3 Prepare control tubes of 10 mL of LETH per organism.

12.4 Inoculate each tube with 0.1 mL of the test organism suspensions, vortex thoroughly and plate in triplicate on the appropriate agar. Incubate plates as described in the test procedure.

12.5 Count the plates and compare the recovered counts from the sample plates to those of the control plates.

12.6 There should be an expected organism recovery of not less than 70%. If the organism recovery is less than 70%, repeat the test using another neutralizer broth or method at the discretion of the study director. If recovered counts are >100 CFU, the neutralization procedure should be repeated with an adjusted titer.

13.0 PRODUCT CATEGORIES

13.1 Table 1 places products into four categories. Knowing the appropriate product category of the test product allows the sponsor to determine which USP acceptance *criteria to apply.*

14.0 USP CRITERIA FOR ANTIMICROBIAL EFFECTIVENESS

14.1 Table 2 explains the criteria for antimicrobial effectiveness for each category. No increase is defined as organism recovery of not more than 0.5 $\log_{10}$ unit higher than the previous value measured.

15.0 EXECUTIVE SUMMARY

15.1 Reason for change

15.1.1 Updated reference.

15.2 Justification of change

15.2.1 USP reference updated.

15.3 Summary of changes

15.3.1 Updated reference.

15.3.2 Added MMF numbers to media.

15.3.3 Optional form pulled out of bundle and given own form number.

15.4 Training Requirements

15.4.1 Read the STP and document the review.

15.4.2 Watch a master perform the procedure.

15.4.3 Perform the procedure satisfactorily with the master watching.

TABLE 1 - PRODUCT CATEGORIES

CATEGORY	PRODUCT DESCRIPTION
1	Injections, other parenterals including emulsions, otic products, sterile nasal products, and ophthalmic products made with aqueous bases or vehicles.
2	Topically used products made with aqueous bases or vehicles, non-sterile nasal products, and emulsions, including those applied to mucous membranes.
3	Oral products other than antacids, made with aqueous bases or vehicles.
4	Antacids made with an aqueous base.

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TABLE 2 - USP CRITERIA FOR ANTIMICROBIAL EFFECTIVENESS

Category 1 Products	
Bacteria:	Not less than 1.0 log reduction from the initial calculated count at 7 days, not less than 3.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast & Molds:	No increase from the initial calculated count at 7, 14, and 28 days.
Category 2 Products	
Bacteria:	Not less than 2.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast & Molds:	No increase from the initial calculated count at 14 and 28 days.
Category 3 Products	
Bacteria:	Not less than 1.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast & Molds:	No increase from the initial calculated count at 14 and 28 days.
Category 4 Products	
Bacteria, Yeast,& Molds:	No increase from the initial calculated count at 14 and 28 days.

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